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a) The repeated 36 amino acid motif of *Chlamydia trachomatis* Hc2 protein binds to the major groove of DNA

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b) Abstract

The gram-negative, obligate intracellular human pathogen, *Chlamydia trachomatis* has a bi-phasic developmental cycle. The histone H1-like *C. trachomatis* DNA binding protein, Hc2, is produced late during the developmental cycle when the dividing reticulate body transforms into the smaller, metabolically inactive elementary body. Together with Hc1, the two proteins compact the chlamydial chromosome and arrest replication and transcription. Hc2 is heterogeneous in length due to variation in the number of lysine rich pentamers. Six pentamers and one hexamer constitute a 36 amino acid long repetitive unit that, in spite of variations, is unique for *Chlamydiaceae*.

Using synthetic peptides, the DNA-binding capacity of the 36 amino acid peptide and that of a randomized peptide was analyzed. Both peptides bound and compacted plasmid DNA, however, electron microscopy of peptide/DNA complexes showed major differences in the resulting aggregated structures. Fluorescence spectroscopy was used to analyze the binding. After complexing plasmid DNA with each of three different intercalating dyes, increasing amounts of peptides were added and fluorescence spectroscopy performed. The major groove binder, methyl green, was displaced by both peptides at low concentrations, while the minor groove binder, Hoechts, and the intercalating dye, Ethidium Bromide, were displaced only at high concentrations of peptides.

Keywords: *Chlamydia trachomatis*; Histone H1-like protein; Hc2; DNA packing; fluorescence spectroscopy; methyl green

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Abbreviations

AA: amino acid; EMBOSS: the European Molecular Biology Open Software Suite; Hc2: *Chlamydia* histone H1-like protein 2; MALDI-TOF: matrix-assisted laser desorption ionization-time of flight instrument;

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c) Introduction

Chlamydia trachomatis is an obligate intracellular gram-negative human pathogen with a unique biphasic developmental cycle in which the small infectious extracellular form with limited metabolic activity, the elementary body (EB) of 0.3 µm, alternates with the larger dividing, intracellular form, the reticular body (RB) of 1 µm [1]. EB can infect genital tract and conjunctival epithelial cells. They attach to the surface of the cells at which point they secrete the translocated actin-recruiting phosphoprotein, TARP, which mediates phagocytosis and recruits actin [2,3]. Upon uptake in an intracellular vesicle, the chlamydial inclusion, EB transforms into the metabolically more active RB, protein synthesis is initiated and the inclusions are transported to the perinuclear space [4]. Early after uptake, chlamydial synthesized proteins of the inclusion membrane protein (Inc) family are secreted by the type 3 secretion system (T3SS) and inserted into the inclusion membrane where they promote inclusion fusion [5]. After multiple rounds of replication, RB are converted into EB. This transition is accompanied by synthesis of late cycle proteins, of which two are cystein-rich outer membrane proteins (Omp2 and Omp3) [6] and two are histone H1-like DNA and RNA binding proteins [7]. During the transition, the outer membrane is cross-linked by disulfide bounds, the diameter of the chlamydiae is reduced from 1 µm to 0.3 µm, the nucleoid is condensed, transcription and replication are arrested and the infectious EB are released through inclusion burst [8].

The two histone H1-like proteins, Hc1 and Hc2, are encoded by *hctA* and *hctB*, respectively [7,9]. Both proteins are capable of condensing both DNA and RNA into tightly packed spheres and arrest replication and transcription [10–12]. Both proteins are abundant in the EB, where Hc1 constitute 6% of the total protein equal to one molecule of Hc1 per 37 bp of the genome [13]. Based on genome analysis, Hc2 is found in all members of *Chlamydiaceae*, whereas in other genera, though proteins with similar amino acid

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composition were found, the very regular presence of repeats was not observed. Thus, Hc2 is believed to be ubiquitous in *Chlamydiaceae* [14] . While Hc1 is genetically stable in all *C. trachomatis* serovars, Hc2 varies in size between serovars [9] and the size variation is caused by variation in the number of repeated elements within the *hctB* gene [14]. The repeated elements consist of 36 amino acids (AA) of which many are positively charged residues. Each element is composed of six pentamers and one hexamer in which both AA substitutions and deletions result in a high number of variants among *C. trachomatis* isolates. The sequence variation makes Hc2 suitable for phylogenetic analysis [14] and is included in a multilocus sequence typing scheme for genotyping of *C. trachomatis* [15] but so far it has not been possible to link variants to clinical disease [12].

Within the repeat [14] the positively charged residues are evenly distributed with two positively charged residues: lysine (K) and arginine (R) separated by three amino acids of which many are either polar uncharged or hydrophobic residues alanine (A), threonine (T) and valine (V). In addition, two prolines (P) are present. Prolines provide conformational rigidity to a secondary helical structure with a kink of the α-helix [16]. To analyze the importance for DNA binding of conserved primary sequence of the repeated 36 AA element of Hc2 we synthesized by peptide synthesis the 36 AA peptide (Hc2rep) and a 36 AA peptide in which the sequence was randomized (Hc2scrbled). The two peptides were analyzed for their DNA-binding capacity by a gel shift assay and electron microscopy. Fluorescence spectroscopy was used to determine where on the DNA helix the peptide bound.

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d) Materials and Methods

2.1 Peptide synthesis

The Hc2-peptide of 36 AA residues, Hc2rep, Accession number GeneBank: ADD14374 aa 61-96 [14] is shown in Fig. 1A. The Hc2rep sequence was pseudorandomized using EMBOSS software to create the Hc2scrambled peptide sequence (Fig 1B). The peptides were synthesized using Fmoc solid-phase-peptide synthesis on an automatic ABI 433 synthesizer (Applied Biosystems, Waltham, MA, USA) according to Holm et al. [17]. The peptides were made with a terminal amide, mimicking an internal peptide bond. The mass of each synthesized peptide was verified by mass spectrometry using an Autoflex matrix-assisted laser desorption ionization-time of flight instrument (MALDI-TOF) (Bruker Daltonics, Bremen, Germany).

2.2 Bioinformatic

Secondary structure predictions Garnier, Osguthorpe and Robson [18] and Helical wheels for the peptides Hc2rep and Hc2scrambled were performed using ”the European Molecular Biology Open Software Suite” EMBOSS [19].

2.3 Gel shift assay

pBluscript SK+ (Stratagene, La Jolla, CA, USA) plasmid DNA was purified from *Escherichia coli* XL1-blue (Stratagene) using cesium chloride gradient centrifugation. DNA concentration and purity were determined by UV scan 220-300 nm (Hitachi, Tokyo, Japan) [20]. Plasmid DNA at a concentration of 33.3 µg/ml was mixed with the Hc2rep peptide and with Hc2scrambled peptide, respectively, in order to obtain final concentrations of peptide of 0, 3.1, 6.2, 12.5, 25 and 50 µg/ml (peptide/DNA ratios of 0, 0.09, 0.19, 0.38, 0.75, 1.5, respectively) in PBS, incubated at 37 °C for 5 min and subjected for electrophoresis

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in a 0.7% agarose gel [12]. The experiments were repeated twice. The gels were scanned and analyzed by ImageJ [21].

2.4 Electron microscopy

Samples with pBluescript SK+ plasmid DNA (33.3 µg/ml) and Hc2rep/Hc2scrambled (0, 3.1, 6.2, 12.5, 25 and 50 µg/ml) in PBS were prepared and incubated at 37 °C for 5 min in order to obtain peptide/DNA ratios of 0, 0.09, 0.19, 0.38, 0.75, 1.5, respectively. Aliquots of the samples were mixed with spermidine buffer [22] and mounted for 5 min onto 400 mesh copper grids coated with a glow discharged carbon film. The grids were then rinsed in double distilled water and dehydrated in increasing concentrations of ethanol at 25, 50, 75 and 96%, blotted dry and rotary shadowed with tungsten wire at vacuum. Electron microscopy was carried out at 60 keV using a JEM 1010 electron microscope (JEOL, Eching, Germany). Images were obtained using a KeenView digital camera (Olympus, Center Valley, PA, USA). For size determination a carbon replica grid (2160 lines/mm) was used. The experiments were repeated three times.

2.5 Fluorescence spectroscopy

Fluorescence spectroscopy was performed twice without stirring and once with magnetic stirring using a QuantaMaster 400, PTI (Photon Technology International Canada, Ontario, Canada) equipped with a xenon lamp using a 1.0 cm light pathway quartz cell. Three different dyes were used for the steady state fluorescence experiments: Hoechst 33342 (Thermo Fisher Scientific, Waltham, MA, USA), Methyl Green (Sigma-Aldrich, St. Louis, MO, USA) and Ethidium Bromide (Merck Millipore, Billerica, MA). Methyl green (1 mg/ml in 0.1% acetic acid) was extracted 3 times with chloroform to remove impurities before the steady state measurements [23].

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Excitation wavelength of 343 nm was used for Hoechst 33342 (Thermo Fisher Scientific) and emission spectra were obtained from 375-600 nm. pBluscript SK+ plasmid DNA was added to obtain a concentration of 0.32 µg/ml in a final volume of 2.8 mL and scanned 6 minutes after addition. Subsequently, 2 µL aliquots of Hc2rep or Hc2scrambled (stock solution at 0.8 mg/mL) were added with regular intervals of 6 min in order to achieve the Peptide/DNA ratios (table 1), after which the emission spectra were recorded with a scanning rate of 1 nm/s.

The same experimental procedure was applied for Methyl green (1.4 µg/ml) and Ethidium Bromide (0.5 µg/ml), using an excitation wavelength of 633 nm (emission: 640-800 nm) [24] and 471nm (emission: 500-700 nm) [25], respectively. As a dilution control, 2 µL of PBS 1x were added successively instead of Hc2rep or Hc2scrambled with regular intervals of 6 min.

The relative fluorescence intensity at the maximum peak was then calculated for each protein/DNA ratio tested and plotted for each of the dyes tested.

2.6 Data treatment

The fluorescence emission spectra of each dye, without DNA, were subtracted from the fluorescence emission spectra obtained for the conditions stated in Table 1. The emission signal was then smoothed (10 points smooth) in Origin 8.1 (OriginLab Corporation, Northampton, MA, USA) and the relative fluorescence of all the emission spectra calculated in Microsoft Excel 2010 (Microsoft Corporation) considering the fluorescence emission maximum of the dyes with DNA but no protein the highest emission (100%). The relative fluorescence of each aliquot at the maximum intensity peak was then plotted for all the dyes in Origin Pro 8 and fitted according to a linear model ($f(x) = a + b*x$), where a is the value of y-intercept of the line and b is the slope. a was fixed at 100, the root mean square error was

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182 calculated as well as the parameter values and corresponding errors. The parameters obtained
183 for the linear fit and the corresponding uncertainties are displayed in Table 2.
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e) **Results**

3.1 Bioinformatics determination of the structure of the C. trachomatis Hc2rep

The 36-mer Hc2rep peptide is composed of only six different AA (11 K, 10 A, 6 V, 4 T, 3 R and 2 P). Its secondary structure was analyzed by Garnier, Osguthorpe and Robson prediction [18]. The structure of the Hc2rep 36-mer peptide was predicted to form an uninterrupted α-helix (Fig. 1A). Using the pepwheel program on the Hc2rep (Fig. 1C) it was seen that the positively charged AA, R and K, are evenly distributed around the α-helix, which also is the case for the remaining AA, A, V, T and P. The Hc2scrambled peptide has the same AA composition as the Hc2rep peptide but with a pseudorandomized sequence. The analysis of the secondary structure by Garnier, Osguthorpe and Robson prediction (Fig 1B) showed that the Hc2scrambled peptide was predicted to form an α-helix, interrupted with turns and coils. Using the pepwheel program on Hc2scrambled (Fig 1D), it was observed that there was a different distribution of the positively charged AA, R and K, and that there was no repeated pentamers.

3.2 Gel shift assay

To determine whether the Hc2rep peptide and the Hc2scrambled peptide could bind to DNA, plasmid DNA (33.3 µg/ml) was mixed with decreasing amounts of each of the peptides and complex formation was analyzed by a gel shift assay. Results are shown in Fig. 2. In lanes 0 no peptides were added to the DNA, and three DNA bands are seen: supercoiled, covalently closed circular (CCC, lower band), nicked open circular (OC, upper band) and linear (L) DNA (middle band) (Fig. 2, inserted at the right side). For both peptides, at 1.5 and 0.75 weight ratio peptide/DNA, no DNA entered the gel, but was retained in the slots, indicating that all DNA was complexed with the peptides. The DNA bands appeared bright in the gel slots when complexed with Hc2rep but with a fainter intensity when complexed with

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Hc2scrambled. At a ratio of 0.38 Hc2rep/DNA, three bands of DNA are entering the gel as supercoiled (CCC, lower band), nicked (OC, upper band) and linear (L) DNA (middle band). However, the bands are fuzzy indicating that the DNA molecules were complexed with various amounts of peptide (Fig. 2, lanes 0.38). This indication was also supported by a faint band observed at the gel slot where part of the DNA appeared to be retained. Similar observations were made for Hc2scrambled/DNA at these ratios. At ratio of 0.19 and 0.09, the DNA bands are increasingly distinct, more defined and no DNA was retained in the slots of the gel. However, it is seen that the lower bands on the gel migrated to a higher position than seen when no peptides were added (Fig. 2, lanes 0). By gel scan there was no indication of a preferential binding of peptides to any of the plasmid forms (CCC, OC or L). The gel scan indicated, however, that the Hc2scrambled peptide was able to complex with DNA (all three forms) better than the Hc2rep peptide (1.5 times better at ratio 0.38; and 1.9 times better at ratio 0.19) as also indicated by visual inspection of the gel (Fig. 2).

3.3 Electron microscopy

To determine how the complex formation between the Hc2rep peptide and the plasmid DNA appeared, electron microscopy was performed (Fig. 3). Decreasing amounts of Hc2rep peptide were added to plasmid DNA, and the complexes were visualized following dehydration and rotary shadowing [22]. At ratio of 1.5 and 0.75 of Hc2rep/DNA, complexes were found in large aggregates seen as tight centers of various sizes with an average of 195 nm in diameter (from 50 to 340 nm in diameter) (Fig. 3A, white arrow), from which loops of DNA were seen (white arrowhead), (Fig. 3A). Other central structures were more elongated (100 x 300 nm) with indication of being built up of twisted DNA strands (black arrow, Fig. 3B). DNA strands (white arrowhead) were seen to protrude from the elongated center. No DNA molecules were seen in the background indicating that at this peptide/DNA ratio all

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DNA was complexed with peptides in agreement with Fig. 2 where at this peptide/DNA ratios no DNA was entering the gel.. There was no clear structure in the aggregated centers but clearly each center had been complexed with a high number of plasmid DNA molecules. At ratio of 0.38 the DNA was complexed with the peptide, forming coiled, elongated central structures (from 200-50 nm) from which loops of DNA could be seen (black arrow, Fig. 3C). In these elongated central structures, it appeared that there was a macrolevel coiling of various numbers of DNA strands. A high number of DNA loops were seen protruding from the elongated centers (Fig. 3C white arrowhead). Similar elongated, coiled central structures (black arrow) appeared at ratio of 0.19 of peptide/DNA (110 – 70 nm), from which loops of DNA could be seen. In addition, supercoiled DNA molecules where seen (Fig. 3D, black arrowhead). At ratio of 0.09 the central structures were shorter (up to 36 nm) and clearly twisted (Fig. 3E, black arrow), forming more loosely organized structures from which loops of both supercoiled (black arrowhead) and linear DNA (L) could be seen protruding. In addition, uncomplexed plasmid DNA molecules could be seen (black arrowhead, Fig. 3E). In Fig. 3F, in which no Hc2rep peptide was added to the plasmid DNA, only uncomplexed plasmid DNA was seen both as supercoiled (Fig. 3F, black arrowhead), open circles and as linear molecules (Fig. 3F), in agreement with the three bands seen by gel electrophoresis (Fig. 2A lane 0).

In Fig. 4 are depicted two electron microscopy images obtained after complex formation between the Hc2scrambled peptide and plasmid DNA at ratios 1.5 (Fig. 4A) and 0.38 (Fig.4B). At ratio 1.5 a smooth compact structure of 1400 x 75 nm (black arrow) is shown, from which a few DNA loops are protruding (black arrowhead) (Fig. 4A, insert). A macrolevel coiling of DNA strands appeared when Hc2scrambled peptide was added to plasmid DNA (Fig. 4B, black arrow) and at this ratio a number of DNA molecules are seen to protrude from the dense central structure (black arrowhead, Fig. 4B) .

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3.4 Fluorescence spectroscopy

Since both the gel shift assay and electron microscopy showed that the Hc2rep peptide was able to form complexes with the plasmid DNA, the next step was to determine how the interaction between plasmid DNA and the peptide was established. Hc2scrambled was used for comparison in these experiments. To determine to which groove the peptide binds, we used a competition assay with three fluorescent DNA binding: the major groove binder, methyl green [23,24]; the minor groove binder, Hoechst 33342 [26,27]; and the intercalator probe, ethidium bromide [28]. Plasmid DNA and the respective fluorescent dye were mixed in a cuvette with magnetic stirring and scanned prior to addition of peptide. Aliquots of peptide were added and after each addition of peptide a new scanning was performed.

To compare the changes in fluorescence between the dyes at increasing amounts of Hc2rep or Hc2scrambled peptide, the maximal emissions were set to 100% without peptide and plotted against peptide/DNA ratio for each dye. The plotted values were then fitted according to a linear model $f(x)= a + b*x$ (Fig. 5). The dynamics of peptide/DNA binding at the tested ratios followed a linear trend with good adjusted root-mean-square (R^2) values (Table 2).

Addition of Hc2rep or Hc2scrambled peptides to the DNA/methyl green (major groove binder), led to a decrease in the relative fluorescence intensity with increase of peptide/DNA ratio, indicating that the peptide had displaced the fluorescent dye from the major groove of DNA. In Fig.5A, it was observed that for the highest peptide/DNA ratio (10.7), the addition of Hc2rep led to a decrease in relative fluorescence intensity of 27% whereas the addition of Hc2scrambled led to a decrease of 35% when compared to the fluorescence decrease of the PBS control with no peptide. Thus, both peptides displaced

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methyl green from the major groove in a similar manner. The linear fittings for both Hc2rep and Hc2scrambled showed adjusted R^2 of 0.999 and 0.997, respectively (Table 2), and the slope obtained for the fitted lines of Hc2rep (-4.2) and Hc2scrambled (-5.0) were statistically different from the slope of the control with PBS (-1.8), determined by the confidence intervals (Table 2).

Similarly, the minor groove binder Hoechst 33342 was analyzed (Fig. 5B). Addition of Hc2rep or Hc2scrambled peptides to the DNA- Hoechst 33342 solution decreased the relative fluorescence intensity by 6% and 11%, respectively. This decrease was lower than what was seen for methyl green, indicating that the displacement of Hoechst 33342 from the minor groove of DNA by Hc2rep and Hc2scrambled peptides was less than what was observed for the displacement from the major groove with methyl green. The slopes obtained from the linear model fitting corroborate these observations, as the slope for Hc2scrambled was more negative than the slope value obtained for Hc2rep (Table 2). Thus, at the highest peptide/DNA ratio a decrease in fluorescence of 8% for Hoechst 33342 compared to compared to 27% with methyl green.

The intercalating dye ethidium bromide (0.5 µg/ml) was excited at 471 nm and scanned from 500 – 700 nm (Fig. 5C). Addition of Hc2rep or Hc2scrambled showed a decrease in relative fluorescence intensity at 600nm, with a higher decrease observed for Hc2scrambled. At the highest peptide/DNA ratio (10.7) a decrease of 1% and 9% was registered for Hc2rep and Hc2scrambled/DNA complexes, respectively. The slopes obtained from the linear model showed a statistical significant difference between PBS/Hc2rep and HC2scrambled (Table 2).

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307 The fluorescence spectroscopy thus showed that methyl green showed the
308 highest displacement with both Hc2rep and Hc2scrambled, while the largest difference
309 between Hc2rep and Hc2scrambled displacement was observed for ethidium bromide, with
310 the highest displacement shown for Hc2scrambled (Fig. 5, Table 2).
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f) Discussion

In the present study we analyzed how the 36-mer repeated part of the *C. trachomatis* histone H1-like protein, Hc2rep, could form large aggregates with plasmid DNA, and found that the peptide preferentially was bound in the major groove of DNA. The 36-mer peptide has a predicted α-helix structure. As one α-helical turn is made up of 3.6 AA the 36-mer peptide has 10 turns. The peptide is rich in the positively charged AA, R and K, which can interact electrostatically with the negatively charged phosphates of the DNA backbone, and the hydrophobic AA, A and V, known to stabilize the α-helix in short peptides in aqueous solutions [29], similarly distributed over the α-helix. Within the 36-mer peptide Hc2rep (Fig. 1A), a pronounced symmetry is seen: two positively charged AA are separated by three uncharged or hydrophobic AA so that each 36-mer peptide is composed of six pentamers and one hexamer [14]. In contrast to the helical wheel analysis of Hc2rep, that showed the positively charged AA to be evenly distributed around the α-helix, the Hc2scrambled peptide had an uneven distribution of positively charged AA and disruption of the predicted α-helix (Fig. 1 B and D)

Large aggregates were formed when plasmid DNA was mixed with high peptide concentrations (Fig. 2-4), and since interactions between the positively charged AA and the sugar-phosphate backbone of the DNA is largely independent of the base sequence [30], a single 36-mer peptide molecule must cross-link several DNA molecules.

At high peptide concentrations, large aggregates of peptides/DNA were formed by both peptides (Fig. 2 - 4). It is clear that at these concentrations the peptides are not binding uniformly along a DNA strand (Fig. 3) but rather aggregating many DNA molecules similarly to what is seen when recombinant *C. trachomatis* Hc2 is complexed with plasmid DNA [12]. Also at the lower concentrations of Hc2rep peptide the complexes formed with plasmid DNA are similar in structure to what was seen with the complete recombinant Hc2,

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where cores of coiled DNA molecules were seen [12]. It thus seems that binding of Hc2rep peptides to plasmid DNA results in coiling of several plasmid DNA molecules forming less tightly wound up aggregates when the Hc2rep peptide concentration was reduced (Fig. 3 C-E). Coil-like structures were also observed when recombinant Hc2 was expressed in *E. coli*, and cells expressing Hc2 were analyzed by electron microscopy [9]. Purified nucleoids from such cells were resistant to DNase I degradation, indicating the intimate binding of Hc2 to the *E. coli* chromosome [9]. The Hc2rep AA sequence is highly similar to part of the AA sequence of the 26 kDa lysine and alanine rich protein of *Chlamydia muridarum* [31]. In their paper Perara et al. [31] suggested that the regular spacing of prolines within the penta-peptide repeat region would result in a kinked helical structure that would assist the fit into the major groove of DNA, and that this would allow K and R residues to form electrostatic and hydrogen-bonds with the phosphate backbone of DNA [9,31]. This is in agreement with our findings, that Hc2rep predominantly binds to the major groove of DNA.

The results obtained with steady state fluorescence with 3 different DNA-binding dyes, showed distinct degrees of binding according to the type of binding dye used (intercalating, binding to the major groove or binding to the minor groove). Binding of the peptides to DNA led to the displacement of the dye which was translated into a decrease in relative fluorescence intensity. The highest decrease was registered for methyl green indicating that the peptides bound primarily to the major groove of the DNA. Even though dilution plays a role in fluorescence intensity decrease, the controls performed with PBS showed that the registered fluorescence intensity decrease for the both Hc2 rep and Hc2scrambled peptides were due to binding and not just due to dilution effect. The acquired data for the tested ratios could be approximated with a linear trend. A good fitting was obtained for all data in the range.

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Electron microscopy showed a marked difference between Hc2rep and Hc2scrambled in the appearance of DNA complexes (Fig. 3 and 4). In the steady state fluorescence, it was seen that Hc2scrambled affected the intercalating ethidium bromide biding in high peptide/DNA ratios significantly, whereas Hc2rep did not (Fig. 5C, Table 2). Therefore, the different distribution of the AA in the peptide structure led to different behaviors upon binding to DNA, and thus the primary AA sequence had a specific function, explaining why the AA sequence is preserved in the chlamydial species [12].

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g) Conflict of interests

Svend Birkelund, Arne Holm and Gunna Christiansen are shareholders in Loke Holdingselskab, Egaa, Denmark, which provided the peptide for this study.

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j) Figure legends

Fig. 1. Secondary structure prediction A) Hc2rep B) Hc2scrambled. Garnier, Osguthorpe and Robson; Helical wheel C) Hc2rep and D) Hc2scrambled.

Fig. 2. Agarose gel electrophoresis A) Hc2rep/DNA complexes B) Hc2scrambled/DNA complexes. The weight ratio between Hc2rep/Hc2scrambled and DNA are marked above the lanes. Std. *Hin*dIII digested lambda λ-phage DNA. OC: open circular DNA; L: linear DNA; CCC: covalently closed circular DNA. At the right of the figure drawings are shown of OC, L and CCC DNA molecules.

Fig. 3. Electron micrographs of Hc2rep/DNA complexes. A-B**)** Hc2rep to DNA ratio 1.5. C) Hc2rep to DNA ratio 0.38. D) Hc2rep to DNA ratio 0.19. E**)** Hc2rep to DNA ratio 0.09. F) DNA. White arrow: aggregated central structure; white arrowhead: DNA loop; black arrow: coiled elongated central DNA structures; black arrowhead: supercoiled DNA.

Fig. 4. Electron micrographs of Hc2scrambled/DNA complexes. A) Hc2scrambled to DNA ratio 1.5. B) Hc2scrambled to DNA ratio 0.38. Black arrow: aggregated, compact DNA/peptide structures; black arrowhead: protruding DNA loops.

Fig. 5. Relative reduction in fluorescence intensity at emission maximum after addition of Hc2rep, Hc2scrambled or PBS for A) methyl green, B) Hoechts 33342 and C) ethidium bromide. Relative intensities are shown on the y-axis and peptide/DNA ratios are marked on the X-axi

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k) Tables

Table 1. Peptide concentrations (µg/mL) and peptide/DNA ratios used in fluorescence steady state measurements with Hoechst 33342, methyl green and ethidium bromide.

Peptide aliquot added	Peptide concentration (µg/mL)	Peptide/DNA ratio
1	0.6	1.8
2	1.1	3.6
3	1.7	5.4
4	2.3	7.1
5	2.9	8.9
6	3.4	10.7

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507 **Table 2.** Linear fit parameters and respective errors using the model *f(x)= a + b*x* (*a*
508 fixed at 100) of the relative values of maximum fluorescence intensity obtained in each
509 condition tested with for methyl green, Hoechst 33342 and ethidium bromide.
510

Dye	Sample	Slope (b)	Adjusted R^2
Methyl Green	PBS	-1.772 ± 0.032	0.999
	Hc2rep	-4.231 ± 0.052	0.999
	Hc2scrambled	-5.000 ± 0.237	0.997
Hoechst	PBS	-0.665 ± 0.048	0.999
	Hc2rep	-1.286 ± 0.041	0.999
	Hc2scrambled	-1.717 ± 0.034	0.999
Ethidium Bromide	PBS	-1.136 ± 0.120	0.999
	Hc2rep	-1.222 ± 0.027	0.999
	Hc2scrambled	-1.993 ±0.042	0.999

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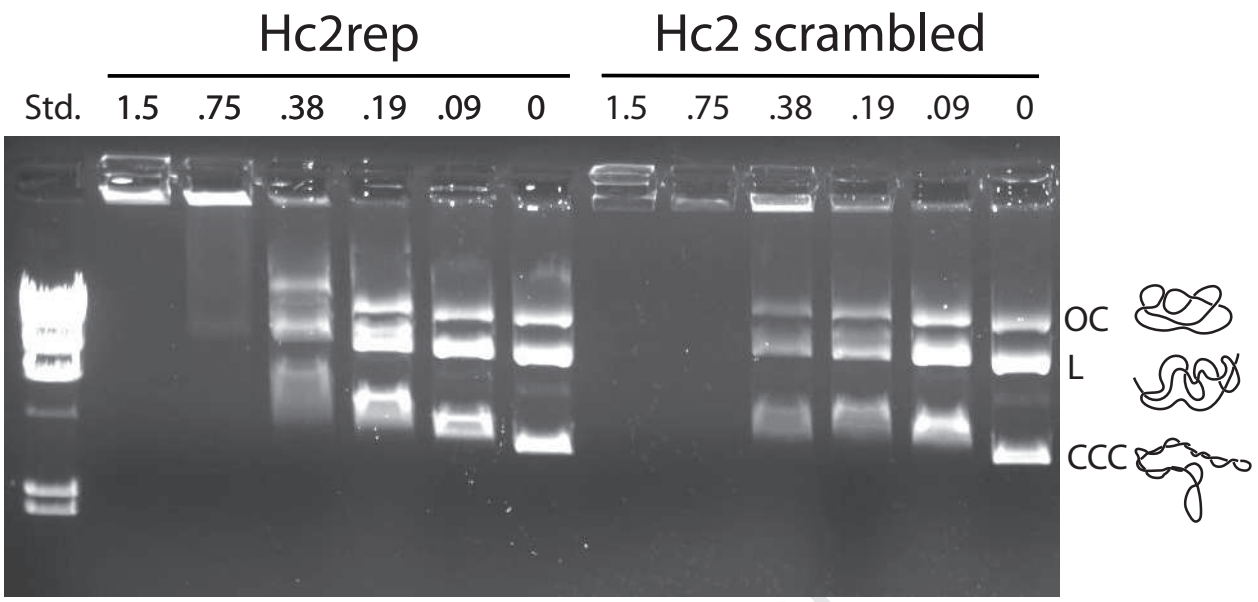

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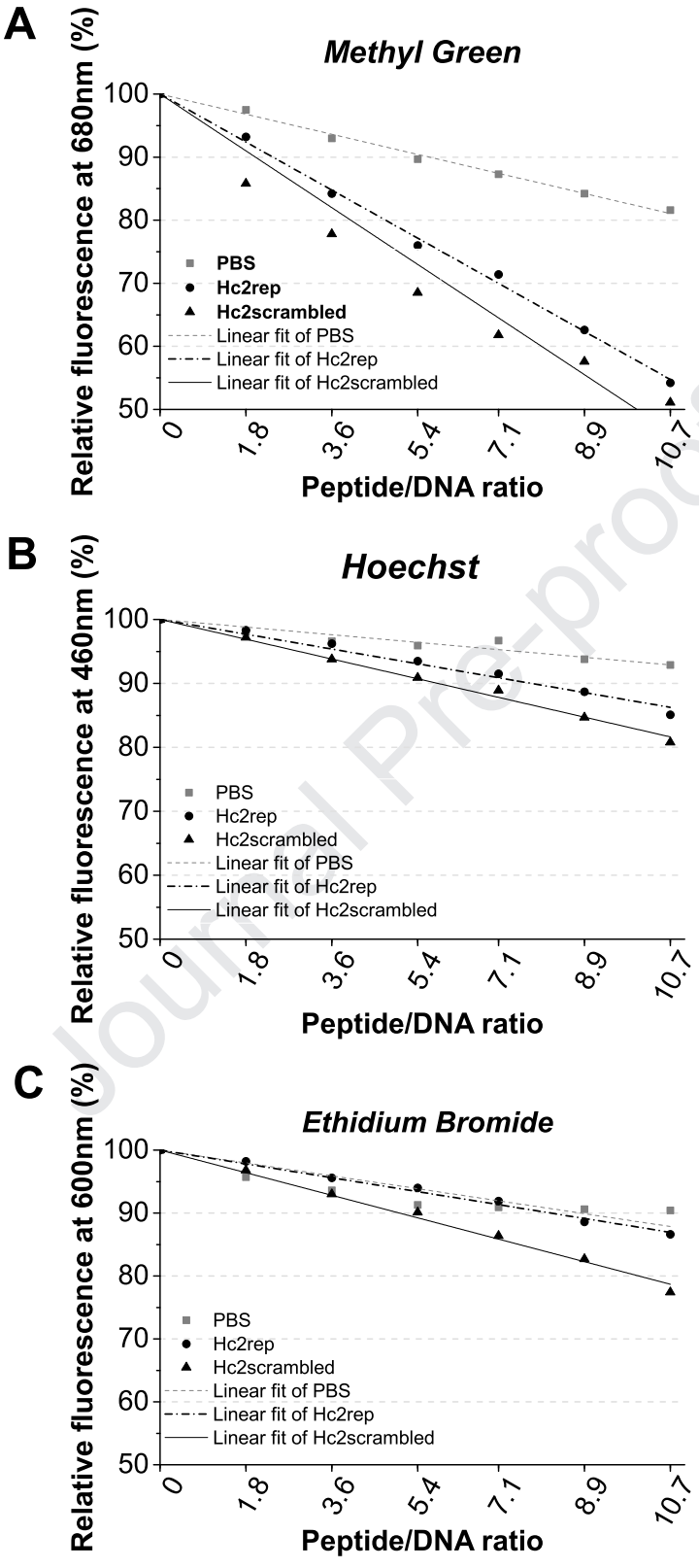

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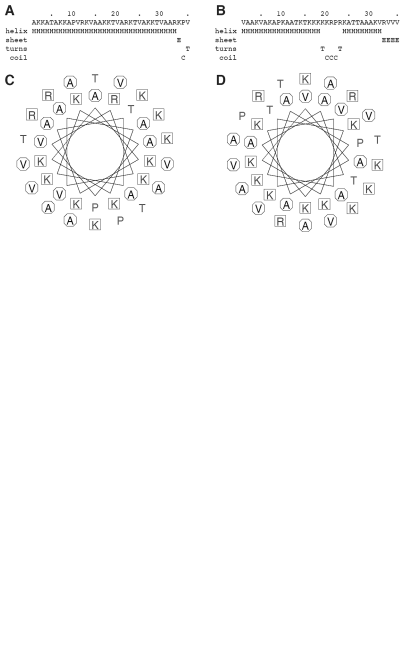

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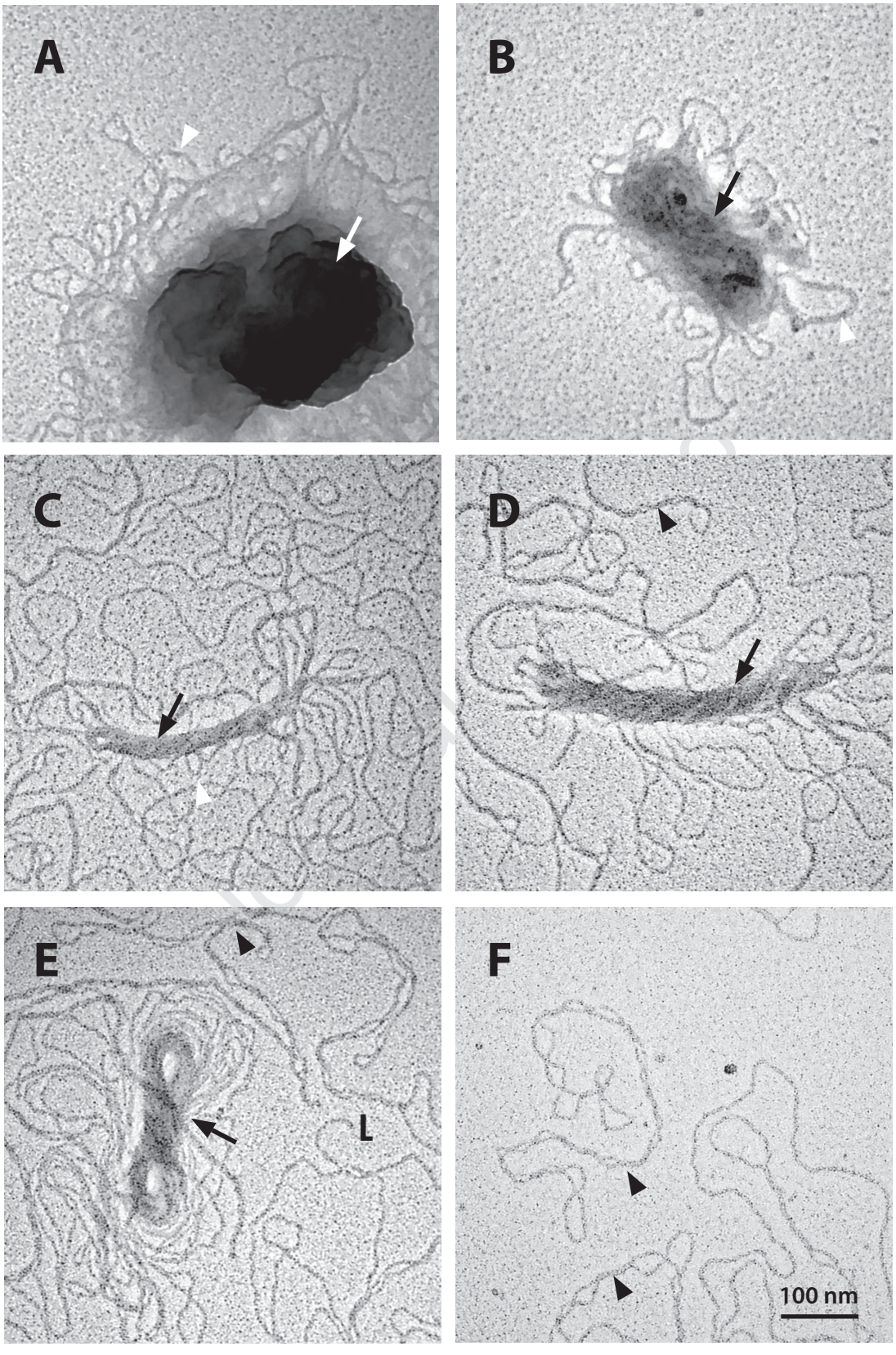
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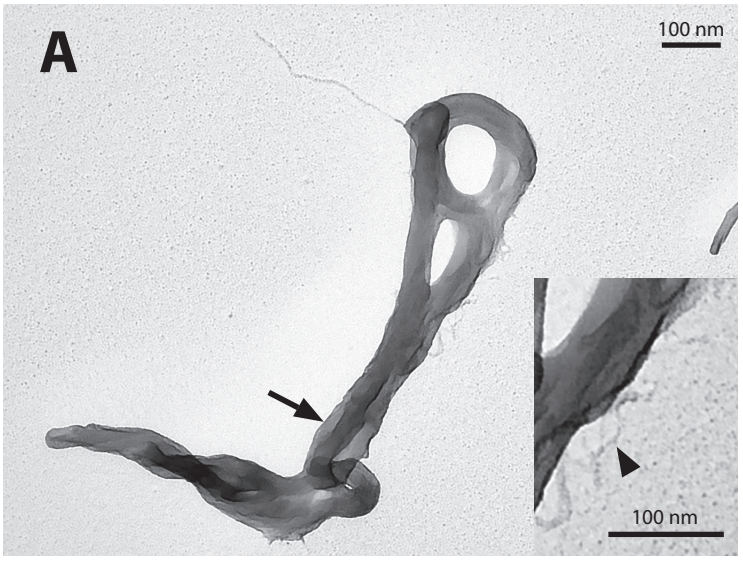

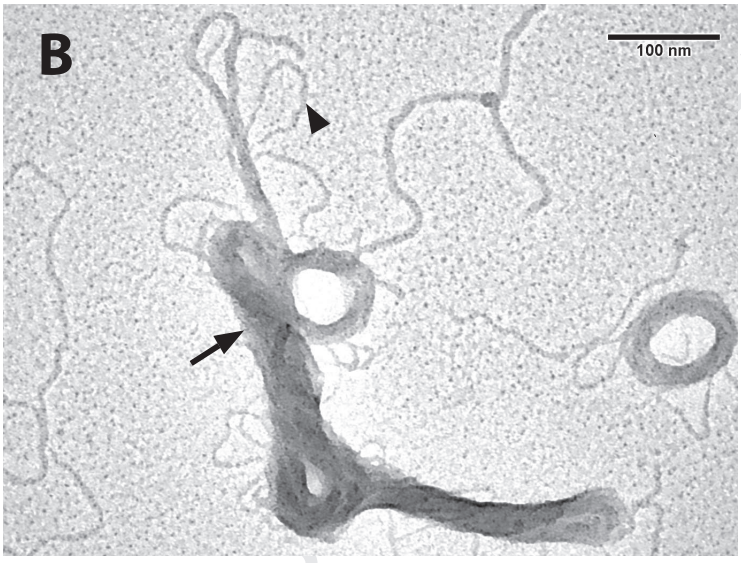

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